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A COMPARATIVE STUDY OF GASTRIC INTRINSIC FACTOR BINDING OF VITAMIN B₁₂

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A comparative study using affinity purified gastric intrinsic factor (IF) was carried out to determine the physicochemical differences between human, rat and rabbit IF. Purified IF from each source was bound to vitamin B₁₂ and taken for proteolysis, paper chromatography, gel filtration, electrophoresis and immunoprecipitation studies under identical experimental conditions. Purified IF complexed with [⁵⁷Co]-vitamin B₁₂ was also used in the experiments. Results of the study indicated inherent differences in the molecular nature of IF from these three sources. The observed differences were (1) in the manner of vitamin B₁₂ binding and release by IF from each source, (2) in the absence of common epitopes on IF from rat and rabbit capable of reacting with polyclonal rabbit antihuman IF-B₁₂ antisera, (3) in the amount of vitamin B₁₂ released when IF-B₁₂ was proteolysed by gastric and pancreatic proteases., and (4) by the lack of immunological cross reactivity and sequence homology between human, rat, rabbit, and hog IF. Release of B₁₂ from proteolysed IF-B₁₂ suggested that vitamin B₁₂ release was not dependent on the action of a specific ileal releasing factor, as had been reported earlier.

Key words: IF-B₁₂ binding, Proteolysis, Species specificity, Immuno complex.

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INTRODUCTION

Gastric intrinsic factor (IF) confers a unique status to B₁₂ as being the only vitamin known to have a carrier mediated transport mechanism in the gastrointestinal tract of man and animals. IF exhibits species specificity, as a result, the clinical use of IF in subjects diagnosed for vitamin B₁₂ deficiency states remains restricted to the use of hog IF. (1-3). Species specificity results due to molecular differences in the synthesis and secretion of genetically variant forms of this carrier protein by the gastric cells of different species. Endogenous differences in the nature of IF molecule produced by each of the three different species, were

therefore investigated under identical experimental conditions. This comparative study is restricted to (a) purifying IF from human, rat and rabbit gastric sources using vitamin B₁₂-sepharose affinity matrix, and (b) assessing the molecular nature of purified IF from each source employing chromatography, electrophoresis and immune reactivity of both native and proteolysed IF-B₁₂ complexes.

MATERIALS

Radioactive [⁵⁷Co] cyanocobalamin, (vitamin B₁₂, 1mci/mg, and 281 mci/mg) and radioactive Na¹²⁵I (4mci) were obtained from the Bhabha Atomic Research Center, Mumbai, India.

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Sephacrose-4B, and Blue dextran were obtained from Pharmacia, Uppsala, Sweden. Cyanogen bromide, pepsin, trypsin, α -chymotrypsin, carboxypeptidase-A and leucine aminopeptidase were obtained from Sigma Chemical Co, Mo, USA. All other chemicals used were of analytical grade from Indian manufacturers.

METHODS

Collection of human gastric juice

Histamine stimulated human gastric juice was collected by nasogastric suction from subjects who underwent this procedure as part of their clinical workup. The collection of gastric juice was made on ice and depepsinized immediately by raising the pH to 10.0 with 5N NaOH, allowing it to stand for 30 min. and then readjusting the pH to 7.5 with 1N HCl. The depepsinized gastric juice was centrifuged at 10,000 x g for 30 min. in a Sorvall RC - 2B refrigerated centrifuge. The supernatant obtained was kept frozen in aliquots for further use.

Preparation of rat and rabbit gastric mucosal extract

Rat and rabbit gastric mucosal extracts were prepared using a procedure essentially as described by Cooper and Castle (4). The animals were given drinking water ad libitum, and their stomach was removed under ether anesthesia. The organ was cut open and washed immediately (using cold 1.15% KCl for the rat stomach, and 0.02M sodium phosphate buffer for rabbit stomach) and the glandular region of the stomach was scraped using a glass slide. The scrapings were taken in 0.1M sodium phosphate buffer, pH 7.5 and homogenised in a Waring blender for 3-5 minutes to obtain a 20% (w/v) homogenate. The homogenate was centrifuged for 30 min at 25,000 x g in an MSE Mistral 6L refrigerated centrifuge at 4°C. The supernatant obtained was

decanted, depepsinised and stored frozen for use as the rat IF source.

Assay for vitamin B₁₂ binding ability:

The vitamin B₁₂ binding ability of human, rat and rabbit IF was determined using the rapid in vitro method described by Gottlieb et al (5). Suitable controls containing protein coated charcoal and free radioactive [⁵⁷Co]-vitamin B₁₂ were set up in parallel. Following centrifugation for 10 min. at 3500 x rpm in a tabletop clinical centrifuge, the control supernatant generally retained less than 1% of the initial total radioactivity of free vitamin B₁₂. The zirconium Phosphate gel (z-gel) at pH 5.0 (6) for estimating the vitamin B₁₂ binding ability of a gastric IF preparation was also carried out. This assay can be used to adsorb IF-B₁₂ complex from a sample quantitatively, to z-gel at pH 6.25 (7) employing type II serum autoantibodies to IF. This method was therefore adopted for the studies reported here.

Protein estimation

Protein in all the samples was estimated by the method of Lowry et al (8).

Purification of intrinsic factor from human, rat and rabbit sources

Intrinsic factor from depepsinised human gastric juice, rat and rabbit gastric mucosal extracts, was purified from the samples prepared and stored frozen earlier, using vitamin B₁₂-sepharose (1 ml gel) affinity chromatography (9). A sample of the IF source was thawed and applied to the gel followed by column washing and elution of the IF protein using established (9) procedures. Purified IF from each source was saturated with vitamin B₁₂ for use in studies reported here.

Polyacrylamide disc gel electrophoresis

Electrophoresis was carried out using the purified protein on polyacrylamide gels (7%) by the method of Baenziger (10). The

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electrophoretic run was conducted in the cold at 4°C using 0.10M sodium phosphate buffer, pH 7.4. Samples taken for electrophoresis contained protein (0.04mg/ml rat IF-B₁₂, 0.66mg/ml rabbit IF-B₁₂ and 0.11mg/ml human IF-B₁₂) in 3% SDS and 1%, 2-mercaptoethanol to which bromophenol blue (0.5%) was added as a tracking dye. Following electrophoresis, gels were stained using 0.25 % coomassie blue in 10% methanol for 18 hr. Destaining was accomplished using 7 % acetic acid in 10% methanol for additional 18 hr.

Proteolysis of the IF-B₁₂ complex

The effect of gastric and pancreatic proteases on purified human, rat and rabbit IF-B₁₂ complexes was assessed by treatment of each sample in 0.1M sodium phosphate adjusted to contain pepsin at pH 1-2, followed 2 hr later with the addition of trypsin, α-chymotrypsin, carboxypeptidase-A and leucine aminopeptidase, at pH 7-8, allowing the reaction to proceed for a further 4 hr. The enzyme to protein ratio was maintained at 1:100 during the reaction at 37°C.

Gel filtration on Biogel P-60

A column (60 cm x 0.6 cm) of Biogel P-60 was prepared at room temperature and equilibrated with 0.1M potassium phosphate buffer, pH 7.4., having a flow rate of 7.5ml/hr. Protein samples containing [⁵⁷Co] vitamin B₁₂ were applied to the column, at a constant flow rate was maintained using a peristaltic pump. Fractions of 1.0 ml were collected employing an LKB Redirac fraction collector and the radioactivity in each fraction was measured using a Packard gamma counter. Void volume (V₀) of the column was determined using 0.5 ml of 1% solution of blue dextran 2000.

Paper chromatography of the IF-B₁₂ Complex

The IF-B₁₂ complexes, native and proteolysed, containing bound [⁵⁷Co]-B₁₂ were chromatographed on a whatman no.

1 filter sheet (size 56.5 cm x 22.5 cm) employing isopropanol/water solvent system (4:1 ratio) at room temperature. The chromatogram was developed for 18hrs by the descending method and dried in air. The dried chromatogram was sprayed with a solution of ninhydrin (0.4%) in acetone and dried (60°C, 15min) to observe the purple products of proteolysis, as well as the native complexes.

Immuno diffusion studies using rabbit antiserum to purified human IF-B₁₂ complex

Polyclonal rabbit antibodies to purified human IF-B₁₂ complex (containing 350 ug IF protein saturated with 8.05 ug B₁₂) was generated in an adult, normal, healthy, male rabbit using a sample (1.0 ml) of purified human IF-B₁₂ complex, mixed with an equal volume of Freund's complete adjuvant and injected subcutaneously into the dorsal skin of a rabbit in several spots. The rabbit was maintained on normal diet for 15 days. A booster dose of the IF-B₁₂ complex (100 ug protein in 0.3 ml mixed with Freund's adjuvant) was then administered to the rabbit on day 16. Blood was collected from the ear lobe of the animal 3rd day following the booster dose, serum separated and stored frozen as the antibody source. Immunodiffusion experiments were carried out using the polyclonal rabbit anti human IF-B₁₂ antiserum against purified human, rat and rabbit IF-B₁₂ forms and hog IF (Sigma), employing the method of Ouchterlony (11).

RESULTS

The amount of total protein in each source applied to the affinity gel matrix, and the amount of purified IF recovered following each column operation is as given (Table 1). The vitamin B₁₂ binding capacity and the specific activity of each source is also given. Differences in the quantitative yield of the IF protein from each source following column operation and differences in their specific activities were observed. The purified IF was however found to be electrophoretically homogenous (Fig.1).

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Human IF was purified 1035 fold, rat IF 1729 fold, and the rabbit IF 116 fold. Purified human IF exhibited three fold greater specific activity than that of the purified rat IF, and purified rabbit IF showed two fold lesser specific activity than that of the purified rat IF. The yield of total vitamin B₁₂ binding capacity of human IF and rabbit IF were closely similar, whereas that of rat IF was 17% lower.

A plot of radioactivity from a sample of [⁵⁷Co]-vitamin B₁₂, native and proteolysed, subjected to paper chromatography and detected based on the radioactivity distribution in one cm width pieces of the paper strips, showed differences in the mobilities of the native and the proteolysed IF-B₁₂ complexes (Table 2).

The Biogel P-60 column elution profile of each IF-B₁₂ complex is as shown in the figure (Fig 2-4). Native IF-B₁₂ of human, rat and rabbit yielded two peaks of [⁵⁷Co]-B₁₂ radioactivity. The position of peak 1 related to protein bound radioactivity and that of peak 2 related to the unbound [⁵⁷Co]-vitamin B₁₂. Proteolysed human and rabbit IF-B₁₂ also yielded two radioactive peaks as for the native complex. Proteolysed rat IF-B₁₂ however, yielded only a single peak of radioactivity. A quantitative decrease in the protein bound [⁵⁷Co]-B₁₂ radioactivity was also observed (Table 3) whenever the proteolysed IF-B₁₂ forms were employed.

Vitamin B₁₂ binding by human gastric juice, rat and rabbit gastric mucosal extracts exhibited first order kinetics. The vitamin B₁₂ binding capacity of each was saturable. Quantitative differences in the capacity of each IF to bind vitamin B₁₂ (tables 1-3) was noted. Differences were observed in their reaction times also. Human gastric juice required 15 min. for saturation of its B₁₂ binding ability (12) whereas rat IF required 5 minutes, and rabbit IF required only 10 seconds (12).

Cross reactivity between the polyclonal rabbit antisera and the IF-B₁₂ complexes of rat and rabbit and the sample of pure

hog IF (Sigma) was not observed during the Ouchterlony immuno diffusion experiments (Table 4). Results of the zirconyl phosphate gel assay however, showed that only the human IF-B₁₂ was capable of being adsorbed onto the gel at pH 6.25 (Table 5).

DISCUSSION

The gastric glycoprotein IF having a molecular weight of 60 kDa and reportedly (13) containing a single site for vitamin B₁₂ binding, when purified from human, rat and rabbit gastric sources employing vitamin B₁₂-sepharose affinity chromatography yielded electrophoretically homogenous IF from each source but having significant differences in their final specific activities. The chromatographic studies established that proteolysed human and rabbit IF retained bound B₁₂ during the gel filtration process. However, fragility of B₁₂ binding by rat IF was observed following proteolysis and gel filtration of the proteolysed rat IF-B₁₂. Nonetheless, retention of bound B₁₂ by human IF following proteolysis was also noted due to the presence of [⁵⁷Co]-B₁₂ radioactivity in the ninhydrin spots that coincided with the protein mobility. Furthermore, electrophoretic mobility differences existed between the native human IF-B₁₂ and the proteolysed human IF-B₁₂ complex. Molecular weight estimates yielded an M_r = 60 kDa for both types of complexes, however.

Z-gel assay detected the loss in type II antibody (7) binding epitope of human IF following the protease action. The presence of the type II epitope in the IF polypeptide is suggested to correlate with the ability of IF-B₁₂ to recognize and bind to its' specific ileal receptors (9). The invitro generation of proteolysed IF-B₁₂ complex, lacking the type II epitope exemplifies the presence of the modified IF-B₁₂ form in the gastrointestinal tract of man and animals.

Cross-reactivity studies did not suggest immunocomplexation of rat, rabbit and hog

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Table 1 Purification of Intrinsic Factor

Affinity step	Sample	Total volume ml	Total activity $\mu\text{g B}_{12}$	Total Protein mg	Specific activity $\mu\text{g B}_{12}/\text{mg protein}$	Yield %	Fold purified
A	Celite filtrate of HGJ	1080	42.12	1890	0.022	100	1
	Guanidine HCl eluate (HIF)	10	23.19	1.05	22.77	57	1035
B	Rat gastric mucosal extract	66.0	0.944	11.55	0.008	100	1
	Guanidine HCl eluate (RatIF)	0.85	0.448	0.0324	13.83	48	1729
C	Rabbit gastric mucosal extract	96.0	2.9	816	0.0036	100	1
	Guanidine HCl eluate (RbIF)	6.2	1.91	4.02	0.466	65	116

Table 2 Paper Chromatography of IF - B₁₂ complex

Sl. No	Sample type	Specific activity $\mu\text{gB}_{12}/\text{mgIF}$	Specific radioactivity $\mu\text{Ci} / \mu\text{gB}_{12}$	Volume applied μl	$[^{57}\text{Co}]\text{-B}_{12}$ (CPM)	R _f
A	Free vitamin B ₁₂ spiked	-	-	20	1000	0.58
B	Native complexes	-	-	-	-	-
1	Human IF - B ₁₂	24	3.5×10^{-4}	50	100,000	0.58
2	Rat IF B ₁₂	13.8	9×10^{-4}	50	20,500	0.59
3	Rabbit IF - B ₁₂	0.5	2×10^{-3}	100	291,000	0.58
C	Proteolysed Complexes	-	-	-	-	-
1	Human IF - B ₁₂	24	3.5×10^{-4}	50	22,300	0.79
2	Rat IF - B ₁₂	13.8	9×10^{-4}	50	41,000	0.66
3	Rabbit IF - B ₁₂	0.5	2×10^{-3}	100	39,000	0.67

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Table 3 Gel filtration using Biogel P-60

Sample type	Volume applied	Total ^{57}Co -B ₁₂ radioactivity applied to column	Number of radioactive peaks eluted
<u>Native complex</u>	(ml)	(cpm)	
Human IF - B ₁₂	0.4	131,000	2
Rat IF - B ₁₂	0.15	42,000	2
Rabbit IF - B ₁₂	0.20	85,400	2
<u>Proteolysed complex</u>			
	0.40	40,000	2
Human IF - B ₁₂	0.15	42,000	1
Rat IF - B ₁₂	0.30	120,000	2
Rabbit IF - B ₁₂			

Table 4 Ouchterlony immunodiffusion studies

Sample	Volume (ml)	Sample protein (ug)	Antisera Protein (mg)	Result
Human gastric juice (well 1)	50	3.0	3.4	Positive
Unproteolysed human IF-B ₁₂ (well 2)	10-50	2.65 – 13.25	4.4	Positive
Proteolysed human IF-B ₁₂ (well 3)	10-50	20 – 10.0	4.4	Negative

Table 5 Zirconium phosphate gel assay

Sl. No.	Sample	Z-Gel at pH 5.0 without antiserum	Z-gel at pH 6.25 with rabbit antihuman IF-B ₁₂ antiserum
1	Human IF-B ₁₂	+	+
2	Rat IF-B ₁₂	+	-
3	Rabbit IF-B ₁₂	+	-

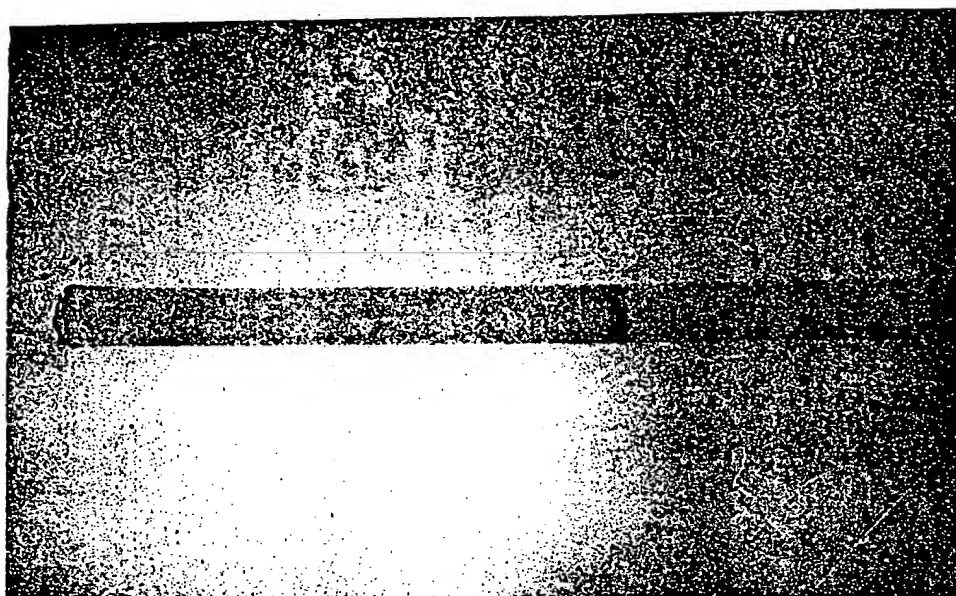


Fig.1 SDS-polyacrylamide disc gel (7%) electrophoresis of affinity purified rabbit IF-B₁₂ carried out for 90 min using 4 milliamps current per tube gel, as described under methods. The coomassie blue stained homogenous protein band is seen (arrow). Affinity purified human and rat IF-B₁₂ yielded homogenous protein bands similarly.

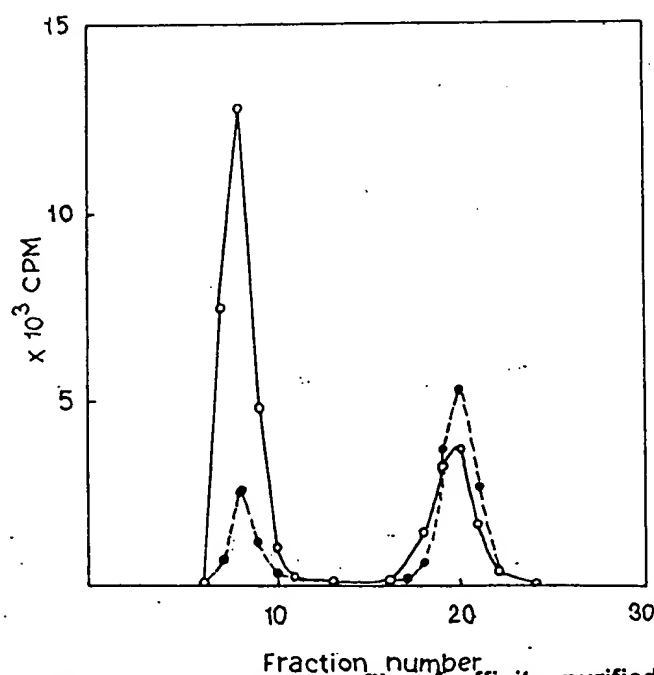


Fig. 2 Biogel-P60 column elution profile of affinity purified human IF-[⁵⁷Co]-B₁₂ based on the B₁₂ radioactivity present in the gel filtered fractions. Gel filtration was carried out as described under methods. Peak 1 represents IF bound B₁₂ radio activity, and Peak 2 represents free B₁₂ radioactivity. Solid line: human IF-B₁₂, Dashed line: proteolysed human IF-B₁₂

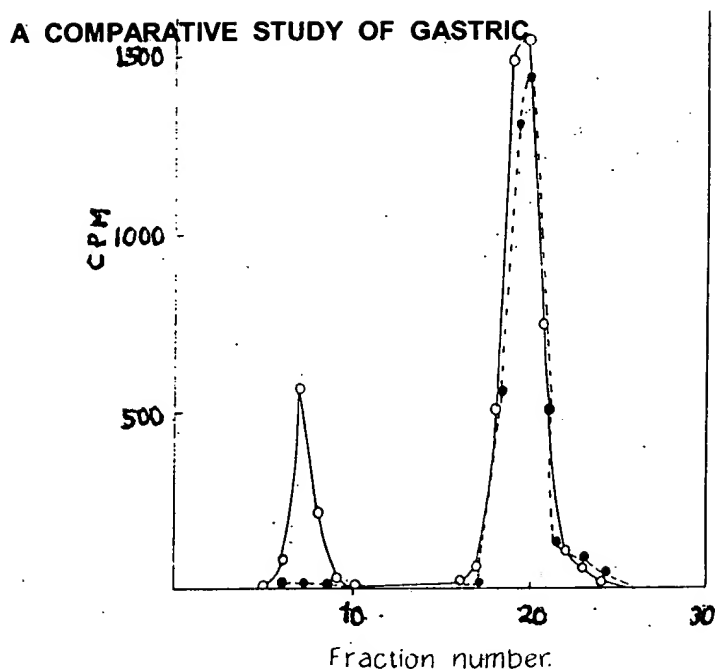


Fig. 3 Biogel-P60 column elution profile of affinity purified ratIF- ^{57}Co -B $_{12}$ based on the B $_{12}$ radioactivity present in the gel filtered fractions. Gel filtration was carried out as described under methods. Peak 1 represents IF bound B $_{12}$ radio activity; and Peak 2 represents free B $_{12}$ radioactivity. Solid line: ratIF-B $_{12}$, Dashed line: proteolysed rat IF-B $_{12}$

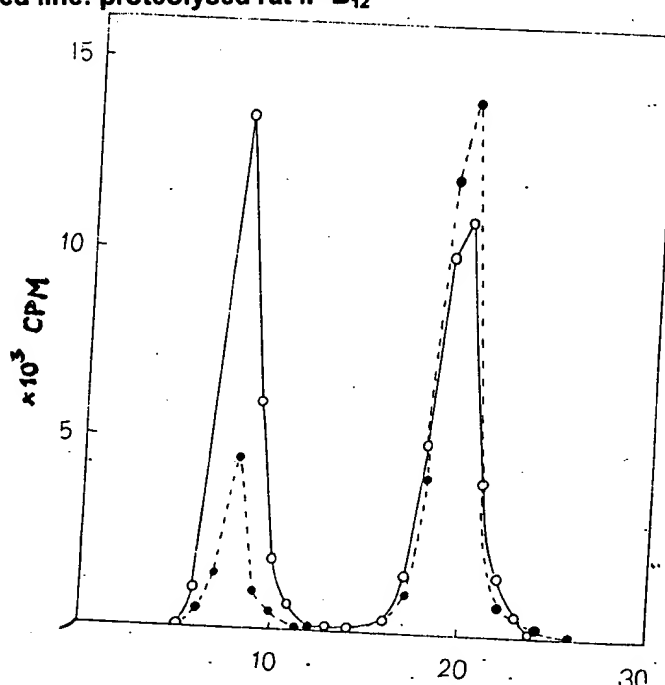


Fig. 4 Biogel-P60 column elution profile of affinity purified rabbit IF- ^{57}Co -B $_{12}$ based on the B $_{12}$ radioactivity present in the gel filtered fractions. Gel filtration was carried out as described under methods. Peak 1 represents IF bound B $_{12}$ radio activity, and Peak 2 represents free B $_{12}$ radioactivity. Solid line: rabbit IF-B $_{12}$, Dashed line: proteolysed rabbit IF-B $_{12}$

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IF with the polyclonal rabbit antihuman IF-B₁₂ antiserum, pointing to the lack of immunological sequence similarities between human, rat, rabbit or the hog IF. Lack of immunoreactivity by proteolysed human IF-B₁₂ with the rabbit antiserum suggested structural changes in the IF-B₁₂ that resulted in the loss of its antibody binding ability.

The observed differences in the IF binding of vitamin B₁₂ may result from, (1) the interaction of other proteins in the gastric IF source with the affinity matrix, reducing the IF binding capacity of the matrix competitively, and thereby affecting the final yield of the purified IF from each source, (2) the nature of non-specific interactions by the glycosyl moiety of IF with other proteins in a sample, may yield conformationally restricted IF polypeptides, remaining incapable of interacting with the B₁₂ ligands of the affinity matrix, and (3) the use of 7.5M guanidine hydrochloride that led to partial denaturation of the IF from different sources and resulted in the reduced vitamin B₁₂ binding ability of the purified IF.

Using partially purified extracts from rat intestines complexed with B₁₂, Rahman (14) observed the kinetics of B₁₂ release to be of an enzymatic type. Ungley (15) proposed and others (16-19) suggested the existence of a 'releasing factor', in the small intestines of man and animals capable of removing vitamin B₁₂ from the IF-B₁₂ complex. Cooper and Castle (4) had confirmed such a proposal using rat intestinal extract and rat IF concentrate. Our studies utilized proteolysis of purified human, rat and rabbit IF-B₁₂ complexes to suggest (a) that structural alterations to IF occurred as a consequence of proteolysis, and (b) that such alterations released bound vitamin B₁₂ (50-70%) from IF.

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